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CONTINUOUS FLOW AND BATCH CULTURES OF
SPHAEROTILUS

M. S. Finstein, et al

Rutgers - The State University
New Brunswick, New Jersey

June 1968

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SPHAEROTILUS**

**Department of Environmental Sciences
College of Agriculture and Environmental Science
Rutgers - The State University
New Brunswick, New Jersey**

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TERMINAL PROGRESS REPORT

RESEARCH GRANT WP-212

CONTINUOUS FLOW AND BATCH CULTURES OF SPHAEROTILUS

by

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Details of illustrations in
this document may be better
studied on microfiche.

June, 1968

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CONTENTS

	Page
Summary	1
Chapter 1. Introduction	2
Chapter 2. Materials and Methods	4
Chapter 3. Utilization of Inorganic Nitrogen Compounds by <u>Sphaerotilus natans</u>	10
Chapter 4. Calcium nutrition of <u>Sphaerotilus</u>	16
Chapter 5. Observations on the Growth Promoting Activity of Spent Sulphite Liquor for <u>Sphaerotilus natans</u>	30
Chapter 6. Attached Growth of <u>Sphaerotilus natans</u> and Mixed Populations in the Continuous Flow Apparatus.....	37
Literature Cited	66
Publication List	70
Acknowledgments.....	71

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SUMMARY

1. Sphaerotilus natans grows, attached, in a continuous-flow apparatus with inorganic nitrogen compounds (NH_4Cl , NaNO_3 , or NaNO_2) as the only source of nitrogen. The growth rate with NH_4Cl -containing medium does not differ from that with media containing glutamate or Casitone as the nitrogen source.

2. Sphaerotilus natans requires calcium for the production of sheaths and probably requires calcium for growth as well, though at a lower concentration. Neither strontium nor barium substitute for calcium. S. natans grows attached to the culture vessels of the continuous flow apparatus even when no sheaths are produced. Tentative evidence is also presented to show that the requirement for calcium is also shared by the manganese oxidizing species, Sphaerotilus discophorus.

3. The calcium in ammonia-base spent sulphite liquor, unlike that in calcium-base spent sulphite liquor, is not available for the growth of Sphaerotilus natans. It is not possible to obtain a mixed slime with Sphaerotilus as the dominant organism in a continuous-flow apparatus fed spent sulphite liquor as the only carbon source.

4. Low concentrations of NH_4Cl and oxygen and high flow rates resulted in attached populations that were dominated by Sphaerotilus in a continuous-flow apparatus fed a medium containing glucose as the only organic nutrient. The environmental factors that allow maximal attached growth of pure cultures of Sphaerotilus do not correspond with the conditions that enrich for Sphaerotilus in a mixed population.

CHAPTER 1.

Introduction

Interest in the filamentous, sheathed bacteria of the genus Sphaerotilus stems, in part, from their ability to grow in large masses in water courses which impair the recreational and other uses of water courses. Sphaerotilus species are also associated with failures in the treatment of waste waters by the activated sludge method in which the filaments of Sphaerotilus have been thought to interfere with the settling of the flocs. Besides their economic significance, interest in Sphaerotilus also stems from their ability of this bacterial genus to produce sheaths in which the individual rod-shaped cells are embedded. Very little is known about the morphogenesis and nature of this characteristic bacterial appendage (35). Another interesting feature of these bacteria is that they are able to grow attached to surfaces, though the exact mode of attachment is not clear.

One of the unsolved problems concerning the nutrition of Sphaerotilus is the ability of these organisms to utilize inorganic nitrogen compounds. A survey of the earlier work led Harrison and Heukelekian (19) to conclude that "there is general agreement that the luxurious growths similar to those found under stream conditions cannot be reproduced in the laboratory with inorganic sources of nitrogen," though some workers did report limited utilization of inorganic nitrogen or utilization under certain conditions. More recently, Mulder and van Veen (31) reported that S. natans could utilize inorganic nitrogen compounds, but not as well as it utilized organic nitrogen compounds. This led A.D. Adamse (Ph.D. thesis, Wageningen, The Netherlands) to suggest this as one of the reasons why S. natans does not dominate in activated sludge treating dairy wastes, the dominant organisms in the sludge, Arthrobacter species, utilizing inorganic nitrogen compounds as well as they did organic compounds.

Our preliminary investigations on the growth of Sphaerotilus in continuous flow cultures suggested that S. natans was, in fact able to readily utilize inorganic nitrogen compounds. Because of the ecological implications

(most of the nitrogen in the natural habitat might be expected to occur in inorganic form), we investigated the problem in greater detail and the results are presented in Chapter 3.

During attempts to grow S. natans in the continuous flow apparatus fed with a dilute, but complex, organic medium, it was found that the medium had to be supplemented with calcium before appreciable attached growth would occur. Since preliminary investigations revealed that calcium profoundly affected the production of sheaths we decided to investigate the nature of the calcium requirement in the hope that this might help us to understand the conditions favoring sheath production. Sheath production is of ecological interest in that the production of sheaths is probably necessary for the physical integrity of the massive proliferations that occur in streams. The problem was also of interest in that very little is known about the mineral nutrition of Sphaerotilus organisms. The results of this investigation are presented in Chapter 4.

The observation on the requirement for calcium led to a comparative study of the growth promoting activities of calcium and ammonia base spent sulphite liquors, the waste waters produced during cellulose manufacture using bisulphites of calcium and ammonia respectively in the chemical digestion of the wood. The discharge of spent sulphite liquors into water courses often results in massive slime infestations of S. natans. A considerable amount of work has been done on the stimulation of S. natans in mixed populations, the most notable being probably that of Amberg and Cormack (3). However, very little (40) is known about the nutritional adequacy of spent sulphite liquors for pure cultures of S. natans. The results of our investigation on this subject are presented in Chapter 5.

In Chapter 6 are presented the results of an investigation to study the effect of varying environmental conditions on the attached growth of Sphaerotilus in the continuous flow apparatus. Also described are the results of an investigation aimed at elucidating the factors that influence the selection of Sphaerotilus from a mixed population.

CHAPTER 2.

Materials and Methods

Strains of S. natans were obtained from lyophilized stocks (13). After revival, the cultures were maintained at 20°C. in CGY broth (32) by semi-monthly transfers. The strain of S. discophorus (strain 35) was obtained from Professor J. L. Stokes. This strain has also been maintained in CGY broth at 20°C.

A hundred-fold dilution of CGY broth with inorganic salts added (hereafter referred to as 1% CGY broth) used in many of the experiments with S. natans had the following composition: Casitone, 50 mg/liter; yeast autolysate (Albimi), 10 mg/liter; glycerol, 50 mg/liter; MgSO_4 , 0.1 mM; phosphate buffer, (K^+ , pH 7.2), 1 mM; in deionized distilled water. In experiments with S. discophorus the glycerol in the above medium was replaced with glucose; the altered medium is referred to as 1% CGY. The phosphate and glucose (as a 20% solution) were autoclaved separately and added to the cool sterile medium. Basal medium, used to make up the synthetic media, contained the following ingredients: MgSO_4 - 0.1 mM; phosphate buffer (K^+ , pH 7.2) - 1 mM; FeCl_3 - 0.01 mM; micronutrient solution (10) - 0.001 ml/liter; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ - 0.005 mg/liter; vitamin B-12 - 5 ug/liter; in deionized distilled water. Various additions to this basal medium are indicated in the text.

The continuous flow apparatus was designed by N. C. Dondero (unpublished). Essentially, it consists of 12 culture vessels through which the medium continuously flows as in a chemostat. It should be emphasized, however, that we are not dealing with "continuous cultures" in the usual sense of the term in that we have attached growth, a situation that must be avoided in conventional continuous cultures in chemostats. Nor do the values of growth yield represent steady states of growth.

Medium was continuously fed into the culture vessels (chemostats) at a flow rate of 180 ml/hr., unless otherwise stated. The culture vessels were fitted with overflow tubes which maintained the volume in the culture vessels at 150 ml. The contents of the culture vessels were stirred with magnetic

stirring bars and aerated (unless stated otherwise) by passing air through the medium through glass gas diffusers (coarse) at a flow rate of 400 ml/hr. The culture vessels were placed in a water bath maintained at 20°C. A diagrammatic representation of a culture vessel is given in Fig. 1. The experimental details of the continuous culture runs were as follows:

The culture vessels, each containing 150 ml of medium, were inoculated with 10 ml of a 48 hr. culture of S. natans growing in 5% CGY broth (5 ml CGY broth (33) diluted to 100 ml with water) on a rotary shaker (180 rev./min.) at 20°C. After incubation for 24 hours, flow of medium through the chemostats was started. After the requisite amount of medium had been fed, the adhering growth was harvested with the aid of a rubber policeman. Either all the harvested cells were used for dry weight determination or suitable aliquots were employed. When aliquots were used, the cell crop, after suitable dilution with water, was homogenized in a "Omni Mixer" (Ivan Sorvall, Inc.) at 800 rev./min. for 1 minute. In all cases the cells were washed twice with water (by centrifugation at about 8000xg) before dry weight (100 C, 24 hr.) or nitrogen determinations. Cellular nitrogen was determined as described by Okrend and Dondero (33) using the method of McKenzie and Wallace (29).

In the experiments described in Chapter 5, where mixed populations were being studied, the inoculum consisted of 5 ml sewage in addition to the culture of S. natans.

Experiments with S. discophorus were done at 28 C. NH_4^+ in culture media was estimated by the method of Ecker and Lockhart (15).

The spent sulphite liquors (SSL) were products of the Crown Zellerbach Corporation, Washington. Prior to use they were adjusted to a pH of 7.2 with KOH. Some characteristics of the two liquors are given in Table 1. The ammonia base liquor had been concentrated by evaporation and was supplied under the trade name "Orzan AL50". Spent sulphite solids refers to the dry substance content of the liquors.

Glassware used in these studies was washed in a detergent, rinsed with concentrated hydrochloric acid, washed thoroughly with hot tap water, soaked in 0.1% disodium

ethylenediaminetetraacetate (EDTA) and then rinsed with deionized distilled water. Deionized distilled water refers to distilled water that was passed through an ion exchange (mixed bed) water purifier (Barnstead).

It should be mentioned that we are still not able to duplicate the growth yields in different experiments even though seemingly identical conditions were employed. However, when all culture vessels were treated identically in the same experiment the coefficient of variation between the culture vessels was about 10%. We have, therefore, made no attempt to compare results obtained in different experiments, and any conclusions arrived at are based on the results obtained in the same experiment.

Fig. 1.

Diagrammatic representation (not to scale) of a culture vessel of the continuous flow set-up. The culture vessels were fabricated by BELICO GLASS CO., Vineland, N.J. "Model 12 AP Dial-A-Pump" (Durrum Instrument Corp., Palo Alto, California) pumps were used. The water bath was made of plexiglas and the temperature was maintained at 20 C by pumping water from a constant temperature bath. "Bell-Stir Multi-Stir" (Bellico Glass Co., Vineland, N.J.) magnetic stirrers were used.

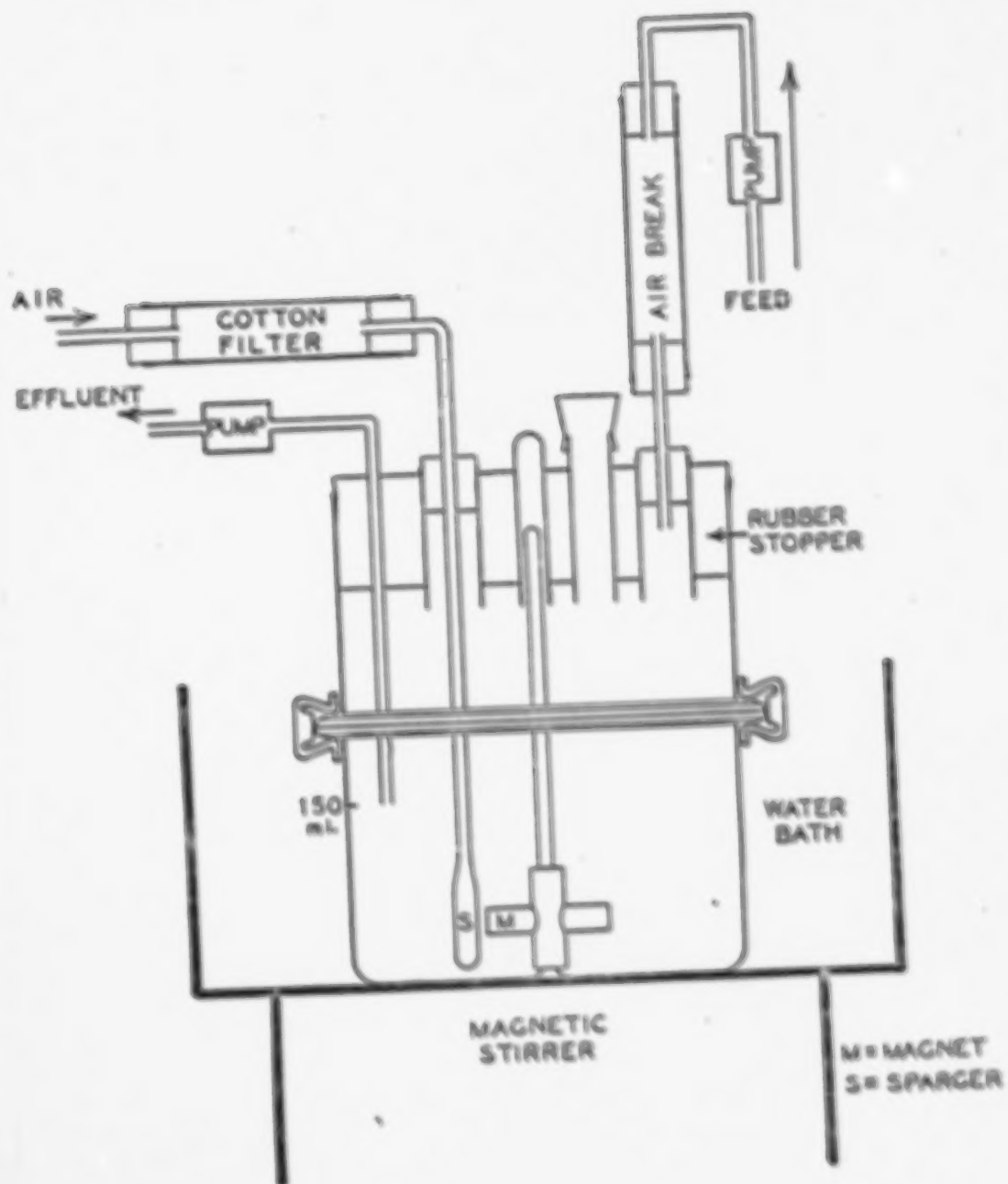


Fig. 1.

Table 1.

Some Characteristics of the Calcium-base and Ammonia-base Spent Sulphite Liquors Used in These Investigations.

	Ca-base liquor	NH ₄ -base liquor
	(g/liter)	
Solids	133	490
Total nitrogen	0	29
Ammoniacal nitrogen*	0	23
Calcium**	1.276	0.312

* Ammonia liberated on adding NaOH.

** Determined by absorption spectrophotometry by S. J. Toth, Dept. of Soils & Crops, Rutgers University.

CHAPTER 3

UTILIZATION OF INORGANIC NITROGEN COMPOUNDS BY SPHAEROTILUSResults:

The results presented in Table 2 show that S. natans, strain 47, is able to utilize all three forms of inorganic nitrogen tested, viz., NH_4^+ , NO_2^- and NO_3^- . The highest concentration of NH_4Cl tested did not seem to have any toxic effect on the growth of S. natans, contrary to the reports of some of the earlier investigators (19) who used batch cultures. Other experiments (not described in this paper) had shown a decrease in the amount of NH_4^+ in the effluent collected during 30 min prior to harvesting cells as compared to the concentration of NH_4^+ in the feed (NH_4Cl in feed: 20 mg/L; NH_4Cl in effluent: 15 mg/L). The limited growth with nitrite and nitrate is probably a reflection of the toxicity of nitrite. However, this point has not been investigated. The other strains of S. natans tested were all able to utilize NH_4^+ (Table 3); their ability to utilize NO_2^- and NO_3^- was not tested.

The experiment described in Table 4 shows that the amount of growth produced with NH_4^+ containing medium is the same as that produced with medium containing sodium glutamate, but more than that produced with a casitone containing medium when the nitrogen compounds were added at identical nitrogen values (about 1.3 mg/L N). The casitone culture was apparently limited by the supply of utilizable nitrogen compounds. This is reflected not only by the decreased growth but also in the lower proportion of cellular nitrogen. Microscopically, the cell chains in all three media were enclosed in sheaths. Interestingly, however, many of the cell chains in the casitone medium were coiled into spirals, some resembling cork-screws. Attempts to induce the formation of such forms in nitrogen deficient batch cultures have not proved successful.

Attempts to obtain growth cultures of S. natans, strain 47, in different media were not entirely successful because of the wide scatter of points, especially in the case of the complex medium. However, it is clear that the growth rate in the NH_4^+ containing medium is not very much different (if at all) from that in either the glutamate or casitone containing media (Fig. 2). It should be noted that the curves were obtained from three different experiments, and that the differences in the lag phase probably are due to variation between experiments which we have not been able to control.

Table 2. Utilization of inorganic nitrogen by S.natans, strain 47.

Nitrogen source added to medium ^a	Dry wt attached growth ^b
	mg
NH ₄ Cl (5 mg/liter)	46
NH ₄ Cl (10 mg/liter)	47
NH ₄ Cl (25 mg/liter)	47
NH ₄ Cl (50 mg/liter)	58
NaNO ₂ (5 mg/liter)	22
NaNO ₃ (10 mg/liter)	37

^aDextrose 50 mg/liter, Vitamin B₁₂ 5 ug/liter, Micronutrient solution (1) 0.01 ml/liter, Na₂MoO₄ · 2H₂O 0.05 mg/liter, mgSO₄ 0.1 mM; CaCl₂ 0.1 mM; PO₄ buffer (K⁺, pH 7.2 1 mM; FeCl₃ 0.01 mM; in distilled water. 9 liters medium were fed through each chemostat.

^bAverage of duplicate culture vessels.

Table 3. Utilization of NH_4^+ by S. natans^a

Strain No.	Dry wt attached growth
	mg
2	45
10	32
32	55
110	41
ffd	56

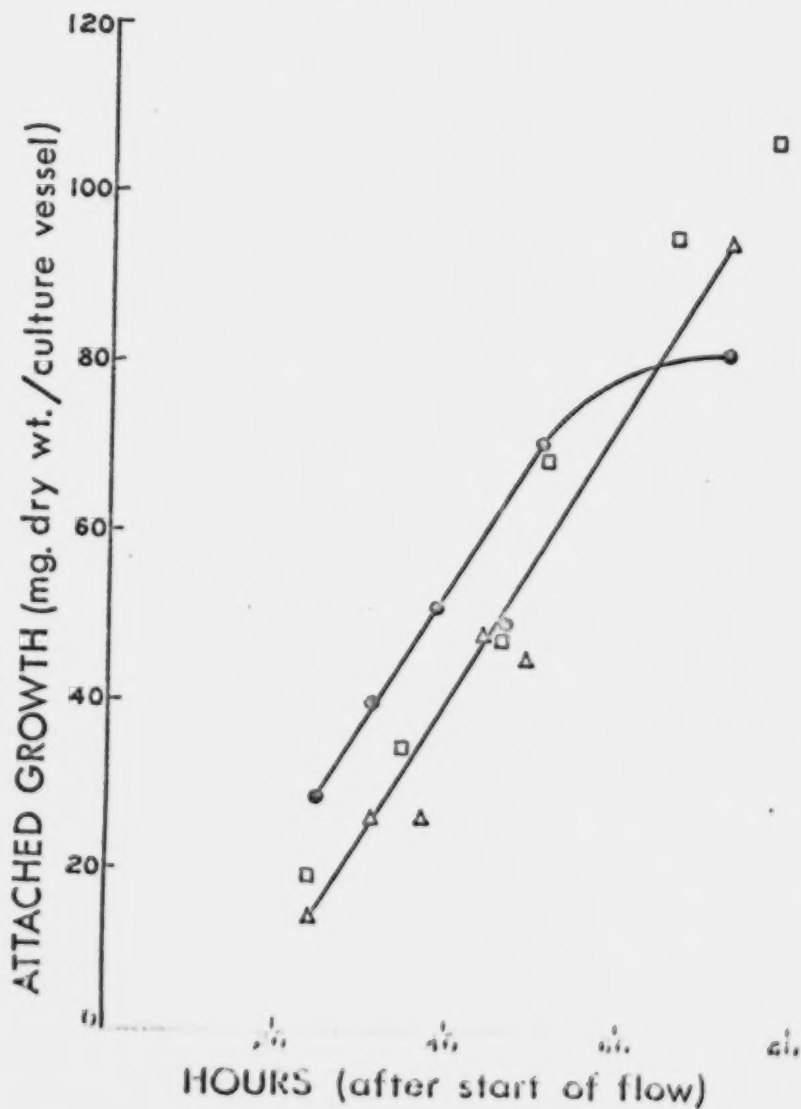
^aThe medium described in Table 2 containing 25 mg/liter NH_4Cl was used. 9 liters of medium were fed through each culture vessel.

Table 4. Growth of *S. natans*, strain 47, in media containing either NH_4Cl , sodium glutamate or casitone as the nitrogen source.

Addition to medium ^a	Attached Growth	
	Dry wt	Nitrogen
	mg	mg
NH_4Cl (5 mg/liter)	56	4.6
Sodium glutamate (15.66 mg/liter)	56	3.2
Casitone (12.42 mg/liter)	27	1.1

^aAs in Table 2. 9 liters of medium were fed through each culture vessel.

Fig. 2. Attached growth of *S. natans*, strain 47, in the continuous flow apparatus fed through media containing either NH_4Cl (25 mg/l); sodium glutamate (30 mg/l) Δ ; or casitone (50 mg/l) + yeast autolysate (10 mg/l) $\square$, as nitrogen sources. The other constituents of the medium are the same as in Table 1. Dilution rate 1.2 hr^{-1} . The curve for the casitone-yeast autolysate medium has not been drawn because of the scatter in points.



Discussion:

The results presented above leave little doubt that S. natans is able to utilize inorganic nitrogen compounds as well as it can utilize organic nitrogen compounds. However, the data fail to reveal the reason why batch cultures give erratic results. They do indicate, however, the usefulness of continuous culture methods in studying "aquatic bacteria" which are normally accustomed to dilute media. A similar approach to the study of marine bacteria was recently reported by Hamilton et al. (18).

CHAPTER 4.

CALCIUM NUTRITION OF SPHAEROTILUSContinuous flow experiments with S. Natans

The experiment described in Table 5 shows the need for calcium for attached growth of S. natans in the continuous flow apparatus fed 1% CGY broth. The type of anion did not have any effect. Very little growth occurs in the absence of added calcium. It should be emphasized that, irrespective of the amount of attached growth, the amount of unattached growth is very small, the turbidity of the effluent being the same as that of the feed. These results have also been obtained with other strains of S. natans (Table 6).

Using various concentrations of calcium chloride it was found that the amount of attached growth in culture vessels fed media containing either 0.1 mM or 0.01 mM calcium chloride was essentially similar (Table 7). However, cultures growing in media with the higher concentration of calcium chloride (0.1 mM) were visibly bulkier and more slimy. When examined microscopically the cultures growing in media containing 0.1 mM calcium chloride showed typically sheathed cells of S. natans, (Fig. 3-a). On the other hand, cultures grown at lower concentrations of calcium were devoid of sheaths (Fig. 3-b). It should be noted that these concentrations are those needed at the detention time used in these experiments. Moreover, the casitone and yeast autolysate used in the medium contain 0.006% (w/w) and 0.025% (w/w) calcium (Ca^{++}) respectively.

Experiments were also done using synthetic media. However, more often than not, no difference in the dry weight of attached growth was obtained by addition of calcium (Table 8) though the effect on sheath formation was always evident. When casitone was added to the synthetic media with no added calcium chloride, growth was suppressed and this inhibition of growth could be reversed by the addition of calcium ions (Table 9).

Batch culture experiments with S. natans: A growth requirement for calcium could not be demonstrated using the synthetic medium of Okrend and Dondero (33). The medium

Table 5.

Effect of Calcium on the Attached Growth of
Sphaerotilus natans, strain 47, in the Continuous Flow
Apparatus

Addition to 1% CGY broth	Total attached growth	
	Dry weight	Nitrogen
	(mg)	(mg)
CaCl ₂ (1 mM)	60	5.48
Ca(NO ₃) ₂ (1 mM)	58	5.1
None	8	0.85

Table 6.

Effect of Calcium on the Attached Growth of
Sphaerotilus natans in the Continuous Flow Apparatus
 Fed 1% CGY Broth

<u>S. natans</u> Strain No.	Dry wt. of attached growth	
	1% CGY broth	1% CGY broth + 0.1 mM CaCl ₂
	(mg)	(mg)
2	8	65
10	8	72
32	12	79
110	11	61

Table 7.

Attached Growth of Sphaerotilus natans, Strain 47
in the Continuous Flow Apparatus at Different Calcium
Concentrations.

CaCl ₂ concentration added to 1% CGY broth	Total attached growth	
	Dry wt.	Nitrogen
	(mg)	(mg)
0	5	0.4
0.0001 mM	5	0.4
0.001 mM	11	0.8
0.01 mM	71	4.7
0.1 mM	83	5.7

Figure 3-a.

Sphaerotilus natans, Strain 47, grown in 1%
CGY broth containing 0.1 M CaCl_2 x 1,500.

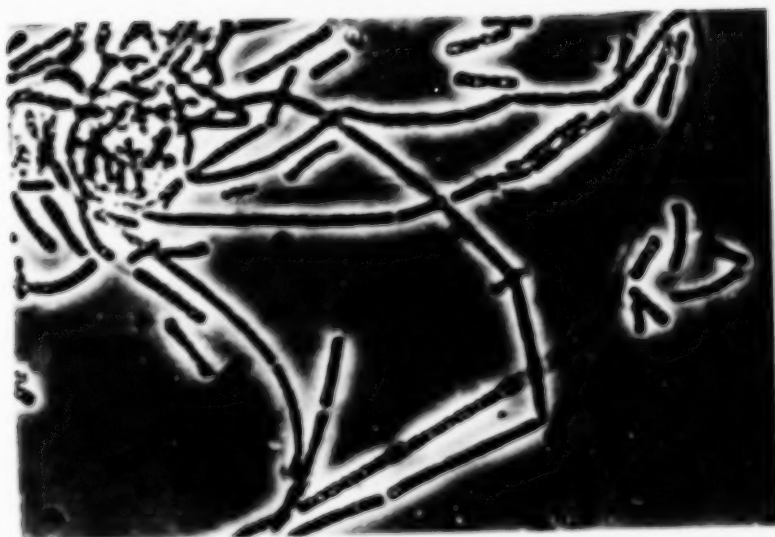


Figure 3-5.

Sphaerotilus natans, Strain 47, grown in 1% CCY broth with no added CaCl_2 at 1,500.

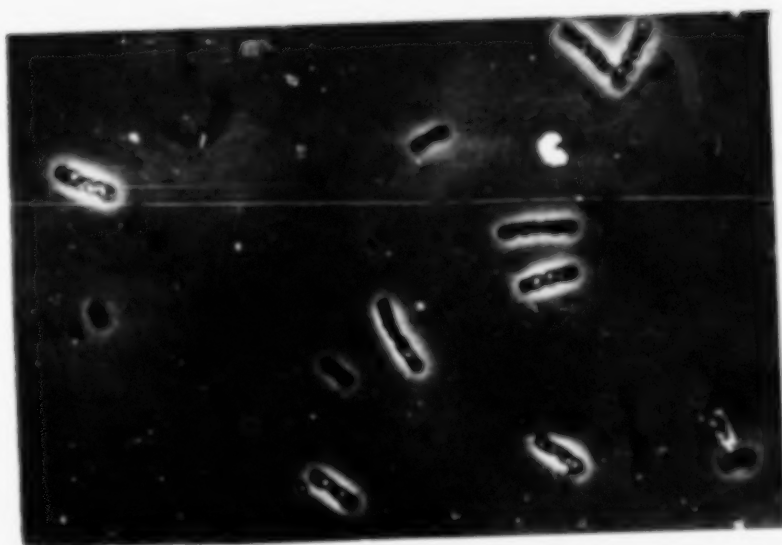


Table 8.

Attached Growth of Sphaerotilus natans, Strain 47,
in the Continuous Flow Apparatus Fed a Synthetic Medium
Containing Different Amounts of Calcium

CaCl ₂ concentration added	Dry wt. of total attached growth		
	A ^a	B ^a	C ^a
	(mg)	(mg)	(mg)
0	104	79	88
0.0001 mM	107	77	
0.001 mM	91	76	97
0.01 mM	101	76	93
0.1 mM	96	84	91

^aA: Basal medium + glycerol, 100 mg/liter + sodium glutamate,
120 mg/liter;

B: Basal medium + glycerol, 50 mg/liter + sodium glutamate,
60 mg/liter;

C: Basal medium + glucose, 50 mg/liter + sodium glutamate
6- mg/liter.

Table 9.

Inhibitory Effect of Casitone on the Attached Growth of *Sphaerotilus natans*, Strain 47, in the Continuous Flow Apparatus, and Its Reversal by Calcium

<u>Addition to medium^a</u>	<u>Dry wt. attached growth</u>
	(mg)
None	80
CaCl ₂ (0.1 mM)	90
Casitone (50 mg/liter)	22
Casitone (50 mg/liter)	
+ CaCl ₂ (0.1 mM)	78

^a Basal medium + glucose, 50 mg/liter + sodium glutamate, 60 mg/liter.

was not specifically treated to render it free of calcium. However, in all instances, cultures from media to which no calcium was added were devoid of sheaths. Growth in 5% CGY broth was completely suppressed by the addition of EDTA (Table 10). This inhibition was reversed by calcium but neither barium nor strontium had any effect (Table 10). Other experiments revealed that magnesium had no effect nor did a mixture of salts (10).

Continuous flow experiments with *S. discophorus*:

A calcium requirement was also evident in the nutrition of the manganese oxidizing species, *S. discophorus* growing in the continuous flow apparatus (Table 11). However, in the case of *S. discophorus* considerable growth occurred in the absence of added calcium. Calcium increased the growth yield. Heavily encrusted sheaths (Fig. 4) were seen only in cultures growing in media supplemented with calcium. The free, rod-shaped cells have a much smaller breadth than the encrusted sheaths. However, unlike in *S. natans* cultures, sheaths were never present in large numbers. Irrespective of whether the cells were sheathed or not, the growth became dark brown from oxides of manganese.

Discussion

The ability of *Sphaerotilus* to grow in the form of free rod-shaped cells rather than in the tubular sheaths characteristic of the genus has long been known (e.g.23). The change from a sheathed to a free living habit can be either phenotypic, controlled by the prevailing environmental conditions, or genotypic. In the former instance it is possible to revert the culture to its sheathed habit by changing the cultural conditions. Gaudy and Wolfe (17) sought to define the conditions that control the formation of sheaths in *S. natans* and noted that of the parameters tested (nature and concentration of carbon source, phosphate concentration, and peptone concentration) only the concentration of peptone had a significant effect on the formation of sheaths. Concentrations of peptone in excess of 0.25% inhibited filament formation. They did not define the nature of this inhibition by peptone. Other workers (42) have reported that smooth colony formation, which is characteristic of sheathless cells, are favored by high peptone concentrations.

Table 10.

Influence of Calcium, Barium, and Strontium on
the Inhibitory Effect of EDTA for Sphaerotilus natans
Growing in Batch Culture

Addition to 5% CGY broth ^a	Amount of Growth	
	EDTA (5 mg/liter) (mg/ml)	No EDTA (mg/ml)
CaCl ₂ (0.1 mM)	0.076	0.08 ^b
SrCl ₂ (0.1 mM)	0	0.079 ^c
BaCl ₂ (0.1 mM)	0	0.079 ^c
None	0	0.074 ^c

^a 100 ml 5% CGY broth/500 Erlenmeyer flask. Incubated
on a rotary shaker (180 rev./min.) for 72 hr. at 20 C.

^b Cells in sheaths

^c No sheaths

Table 11.

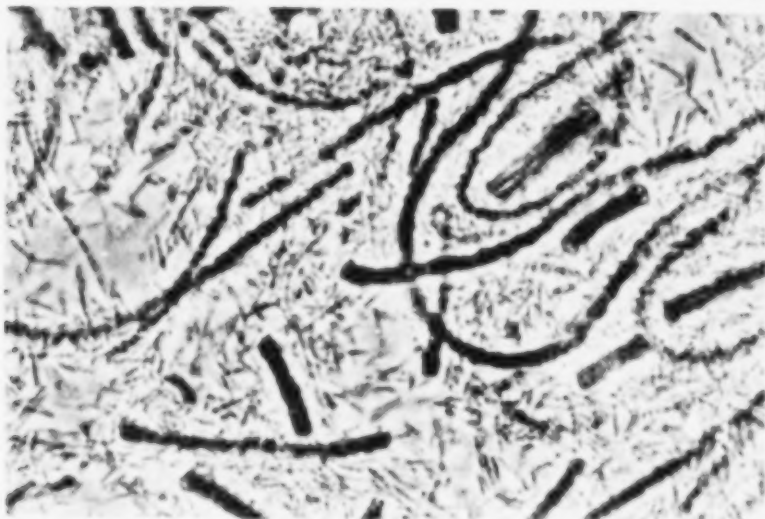
Growth of Sphaerotilus discophorus in the Continuous Flow Apparatus

Addition to medium	Total attached growth	
	Dry wt.	Nitrogen
	(mg)	(mg)
CaCl ₂ (0.1 mM)	95	8.4
+ MnSO ₄ (0.01 mM)		
MnSO ₄ (0.01 mM)	30	3.2

^a 1% CGY broth + FeCl₃, 0.01 mM + micronutrient solution (10), 0.001 ml/liter + biotin, 1.25 µg/liter + thiamine, 50 µg/liter + vitamin B-12, 0.75 µg/liter + Na₂MoO₄, 0.005 mg/liter.

Fig. 4.

Cells of Sphaerotilus discolorus grown in the continuous flow apparatus. Note the much greater diameter of the encrusted sheaths compared to the free rod-shaped cells x 1,500.



Our results show that calcium is required for the formation of Sphaerotilus sheaths. (The change to a non-sheathed habit was phenotypic as indicated by the fact that the sheathless cells reverted to the filamentous form on being cultured in a medium containing adequate supplies of calcium.) This finding probably explains the reports of the influence of peptone concentration on sheath formation. Various amino acids act as metal chelators (1,2) and could possibly be influencing sheath formation by binding calcium. There are reports in the literature (25,27,38) of peptone influencing microbial growth due to its chelating activity. The finding that calcium is required for sheath formation is not entirely surprising in that the ion has been shown to be involved in cell wall morphogenesis, (e.g. 5,14,22). A point of difference is the marked specificity for calcium exhibited by S. natans as is indicated by the experiment using EDTA (Table 10). In Rhizobium the calcium requirement is substituted by strontium (19); in other organisms the requirement is even less specific.

A disturbing feature of the present investigation was the observation that while a calcium requirement could be shown in 1% CGY broth, a nonsynthetic medium, no such requirement could be shown in a synthetic medium. This observation might be explained in the light of the report (16) that calcium deficient conditions cause cells to adhere to surfaces. In fact, we accidentally observed that if the calcium is precipitated out as calcium phosphate, presumably stripping the medium of all calcium, one obtains an almost instantaneous attachment of S. natans cells to the surfaces of the culture vessel. Thus, the attached growth observed in the synthetic media without calcium represents such adhesion followed by growth at the expense of the traces of calcium present as impurities in the medium, but sheath formation does not occur as the process requires higher concentrations of calcium. When casitone is added to such a medium enough calcium is available to prevent adhesion but not sufficient to allow sheath formation and so no attached growth occurs. The free cells are washed away from the culture vessel and do not have an opportunity to multiply and grow unattached in the culture vessels. When calcium is added to such a medium enough calcium is available for sheath formation and the sheaths attach to surfaces and

growth proceeds. This explanation supposes that sheaths are capable of attachment, an assumption commonly made (e.g. 35), though not a proven fact. It should be mentioned, though, that structures on the sheath have been described as being involved in attachment (34). It should also be pointed out that the prima facie hypothesis that the results represent an inhibition of growth by the casitone (26,46) and its reversal by calcium is untenable in view of the fact that the organisms grow successfully in much higher concentrations of casitone, namely in CGY broth.

Though the continuous culture experiments strongly suggest that calcium is also needed for growth of Sphaerotilus, the results are open to more than this one interpretation especially in view of the fact that the amount of unattached growth was not estimated other than noting that the turbidity of the effluent did not differ from that of the feed. However, the EDTA experiment (Table 10) strongly indicates a growth requirement by S. natans for calcium in addition to the requirement for sheath formation. The fact that the requirement for sheath formation, but not the requirement for growth, could be demonstrated in synthetic media indicates that the requirement for the latter function is greater. It is interesting to note that Rouf and Stokes (37) reported that S. discophorus would not grow in a peptone medium unless it was supplemented with a mixture of salts. Our experiments with this species do not permit a conclusion as to its requirement for calcium though they do reveal that calcium is needed for sheath formation in this species as well. That the strain of S. discophorus produced considerable growth in the continuous flow apparatus even in the absence of added calcium in contrast to S. natans is probably a reflection of a quantitative difference between the requirement of the strain of S. natans and that of S. discophorus.

In view of the fact that Sphaerotilus organisms are able to accumulate large reserves of poly-B-hydroxybutyric acid (PHB) (30,31,36,37) it is interesting to point out that the PHB-depolymerases of some bacteria, though not all, are activated by calcium (9,30). S. discophorus PHB-depolymerase is not activated by calcium, however (44).

CHAPTER 5.

OBSERVATIONS OF THE GROWTH PROMOTING ACTIVITY
OF SPENT SULPHITE LIQUOR FOR SPHAEROTILUS NATANSResults:

The effect of SSL on the ability of 1% CGY broth to support attached growth of S. natans in the continuous flow apparatus is given in Tables 12 and 13. SSL-Ca, when added at a solids concentration of 13.2 mg/liter, supplies enough utilizable calcium to allow maximal yields of attached growth; the further addition of CaCl_2 does not increase the amount of attached growth. On the other hand SSL- NH_4 does not supply adequate amounts of available calcium even when added at a solids concentration of 195.8 mg/liter. It is evident from Table 1 that this concentration of SSL- NH_4 has more calcium than does 13.2 mg/liter solids of the calcium base liquor.

Attempts to grow S. natans in a medium containing no organic supplements were not successful. The results are given in Tables 14 and 15. The growth yields are rather low even if one assumes that only 20% of spent sulphite solids are readily utilized by bacteria. The constituents of SSL are given by Vasseur (45). The results in Tables 14 and 15 also indicate that SSL are deficient in vitamin B_{12} . The requirement for this vitamin by S. natans is known (31,33).

Results of experiments to see if S. natans could be preferentially encouraged to grow in the continuous flow apparatus fed a medium containing SSL and inoculated with sewage or S. natans or both are recorded in Table 16. In this experiment, the amount of medium fed through each culture vessel was 18 liters, double that used in other experiments. Appreciable attached growth of the mixed population occurred the amount being nearly three times as great as when Sphaerotilus was inoculated alone. The attached slime produced by the mixed population was not dominated by Sphaerotilus even in the unit inoculated with sewage and S. natans. The mixed population slimes have a greater tendency to slough than do slimes of Sphaerotilus alone. The greater amount of mixed population slime shows, once again, that not all the utilizable substances in SSL are utilized by S. natans. It might be noted that in similar experiments in which diluted sewage (BOD=20 mg/liter) containing glucose (20 mg/liter) was used as the growth medium, Sphaerotilus was the visibly "dominant" organism in the slime.

Table 12. Effect of adding calcium-base spent liquor (SSL-Ca) to 1% CGY broth on the attached growth of Sphaerotilus

Addition to 1% CGY broth	Dry wt attached growth
	mg
None	23
SSL-Ca(13.2 mg solids/liters)	103
SSL-Ca(13.2 mg solids/liters	110
+ CaCl ₂ (0.1 mM)	
CaCl ₂ (0.1 mM) ²	93

*9 liters of medium were fed through each culture vessel.

Table 13. Effect of adding ammonia-base spent sulphite liquor (SSL-NH₄) to 1% CGY broth on the attached growth of Sphaerotilus.

Addition to 1% CGY broth	Dry wt attached growth
	mg
None	15
SSL-NH ₄ (13.2 mg solids/liter)	27
SSL-NH ₄ (13.2 mg solids/liter) + CaCl ₂ (0.1 mM)	110
SSL-NH ₄ (196 mg solids/liter)	56
SSL-NH ₄ (196 mg solids/liter) + CaCl ₂ (0.1 mM)	122
CaCl ₂ (0.1 mM)	102

*9 liters of medium were fed through each culture vessel

Table 14. Effect of glutamate and vitamin B₁₂ on the growth of Sphaerotilus in the continuous flow apparatus fed spent sulphite liquor (SSL)

Addition to medium* (per liter)	Dry wt attached growth	
	A**	B**
	mg	
Sodium glutamate(960 mg)	51	52
Vitamin B ₁₂ (5 ug)	7	1.5
Sodium glutamate (60 mg) + vitamin B ₁₂ (5 ug)	74	75

* SSL, 132 mg solids/ liter + CaCl₂, 0.1 mM + MgSO₄, 0.1 mM + PO₄ buffer (K⁺, pH 7.2), 1 mM; in deionized distilled water. Nine liters of medium were fed through each culture vessel.

**A: Ammonia base SSL; B: Calcium base SSL

Table 15. Growth of *Sphaerotilus* in the continuous flow apparatus fed ammonia-base spent sulphite liquor (SSL-NH₄) supplemented with various nutrients.

Additions to medium* (per liter)	Dry wt attached growth
	mg
SSL-NH ₄ (137 mg)	13
SSL-NH ₄ (137 mg) + dextrose (50 mg)	93
SSL-NH ₄ (137 mg) + sodium glutamate (30 mg)	42
Sodium glutamate (30 mg)	30
SSL-NH ₄ (137 mg) + NH ₄ Cl (10 mg)	9

*CaCl₂, 0.1 mM + MgSO₄, 0.1 mM + PO₄ buffer (K⁺, pH 7.2), 1 mM + Vitamin B₁₂, 5 ug/ liter in deionized distilled water. Nine liters of medium were fed through each culture vessel.

Table 16. Extent of attached growth in the continuous flow apparatus fed a medium containing spent sulphite liquor and inoculated with sewage and/or Sphaerotilus.

Inoculum	Dry wt attached growth
<u>S. natans</u> **	mg 23
Sewage**	63
<u>S. natans</u> and sewage	74

*Ammonia base spent sulphite liquor, 137 mg/liters, vitamin B₁₂, 5 ug/liters, MgSO₄, 0.1 mM; CaCl₂ 0.1 mM; PO₄ buffer (K⁺, pH 7.2) 1 mM; in deionized distilled water. Eighteen liters of medium were fed through each culture vessel.

**Five ml of either settled domestic sewage or a culture of S. natans, strain 47, growing in 5% CGY broth (3).

DISCUSSION

No evidence could be adduced to support the thesis that SSL is an "excellent substrate for Sphaerotilus growth." It is evident from the observation that a glucose medium effectively selects for Sphaerotilus from mixed population, that the experimental conditions involved were otherwise suitable for Sphaerotilus growth. Thus, an explanation for the frequent observation that the discharge of SSL into water courses results in massive Sphaerotilus infestations does not seem to lie entirely in the nutritional suitability of SSL for Sphaerotilus growth. It is interesting to note that slime problems did not arise in the Altamaha River for nearly two years after the discharge of waste waters was begun (28). It is likely that a number of factors are responsible for triggering the sudden outbreaks of slime infestations. SSL have been reported to be deficient in phosphorus (3) and the results reported above show that it is also deficient in vitamin B₁₂. The latter deficiency is probably of minor significance in the natural habitat.

Our observations on the calcium requirement for Sphaerotilus growth would seem to indicate that this might be a parameter that should be considered. The results presented in this paper indicate that in a habitat deficient in calcium, a calcium-base liquor would be more likely to cause Sphaerotilus infestations than an ammonia-base liquor. The small amounts of calcium in ammonia-base liquor, which apparently originated from wood, and occur in organic molecules, is not available to Sphaerotilus. However, in nature, organisms might be present which liberate the calcium and render it suitable for Sphaerotilus growth.

At the time of writing of this paper, Baalarud (6) published results which clearly indicate that the stimulation of Sphaerotilus by SSL is determined, to a large extent, by the quality of the receiving water. It is of interest to note that the water of the river Drans, which supported heavy Sphaerotilus growth, has greater hardness than the water of the river Otra which did not contain Sphaerotilus. However, the waters of the two rivers also differed in other respects.

CHAPTER 6.

ATTACHED GROWTH OF SPHAEROTILUS NATANS AND MIXED POPULATIONS
IN THE CONTINUOUS FLOW APPARATUS

Results:

Effect of nitrogen nutrition: Pure cultures of Sphaerotilus slimes contained different amounts of nitrogen depending on the amount of nitrogen in the medium (Table 17). The amount of nitrogen in the slime ranged from 3.2% to 7.1%. All cultures, irrespective of their nitrogen content, contained normally sheathed cells.

The nitrogen content of mixed population slimes also varied with the amount of NH_4Cl in the medium (Table 18), the maximum (11.4%) being greater than that recorded for pure Sphaerotilus slimes. The types of organism in the slime was greatly influenced by the amount of nitrogen in the medium. At NH_4Cl concentrations of 1 mg/liter and 0.2 mg/liter, the slimes contained large numbers of Sphaerotilus filaments and could be frankly described as a Sphaerotilus infestation (Fig. 5 and 6). However, when the concentration of NH_4Cl was increased (5 mg/liter) the dominance of Sphaerotilus was no longer evident (Fig. 7). It is unfortunate that the results do not permit one to calculate the exact C:N ratio necessary for optimal growth.

The addition of organic nitrogen compounds resulted, in some instances, in an increased amount of attached growth (Table 18) due, no doubt, to the increased amount of available carbon. However, the three compounds tested did not have any selective influence on the growth of Sphaerotilus (Fig. 8 and 9).

Effect of glucose concentration: The weight of attached growth of Sphaerotilus, as was expected, increased with mounting glucose concentrations within the range of concentrations tested (Table 19).

The amount of mixed population slime also increased with glucose concentrations, the increase being linear up to a glucose concentration of 50 mg/liter (Table 20). This concentration is higher than that recorded for pure culture slimes (25 mg/liter). Interestingly, the mixed population

Table 17.

Effect of NH_4Cl Concentration on the Amount of Attached Growth of Sphaerotilus natans in the Continuous Flow Apparatus

Experiment	NH_4Cl concentration in medium ^a (mg/liter)	Attached growth		
		Dry wt. (mg)	Nitrogen (mg) % (w/w)	
I	0.05	4.4	0.18	4.1
	0.1	5.5	0.2	3.6
	0.2	10.6	0.37	3.6
	0.4	17.6	0.64	3.6
II	0.5	26.5	0.86	3.3
	2.5	45	2.23	4.9
	5	44.9	3.2	7.1

^a Added to the basal medium containing 50 mg glucose/liter. The flow rate was 180 ml/hr and 9 liters were passed through each culture vessel.

Table 18.

Effect of NH_4Cl Concentration and Organic Nitrogen Compounds on the Amount of Attached Growth of Mixed Populations in the Continuous Flow Apparatus

NH ₄ Cl concentration in medium ^a	Additional nitrogen source ^b	Attached growth		
		Dry wt.	Nitrogen	
mg/liter		(mg)	(mg)	(%)
0.2	None	13.6	0.46	3.4
1	None	40.3	1.36	3.4
5	None	45.1	3.24	7.2
10	None	49.5	5.62	11.4
10	Casitone	63.1	7.9	12.5
10	Sodium	56	6.64	11.5
	glutamate			
10	L-Methionine	52.8	6.23	11.9

^a Added to the basal medium containing 50 mg glucose/liter. The flow rate was 180 ml/hr and 9 liters were passed through each culture vessel.

^b Added at a concentration of 40 mg/liter of medium.

Fig. 5.

Effect of NH_4Cl Concentration and Organic Nitrogen Compounds on the Nature of the Slime Developing in the Continuous Flow Apparatus.

0.2 mg NH_4Cl /liter of medium

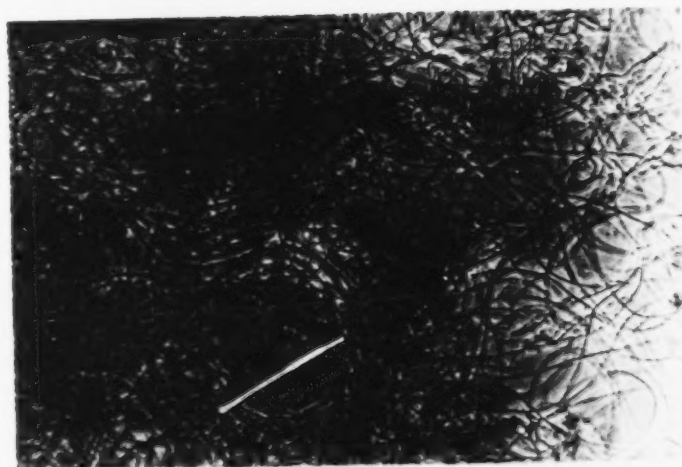


Fig. 6.

Effect of NH_4Cl Concentration and Organic Nitrogen Compounds on the Nature of the Slime Developing in the Continuous Flow Apparatus.

1 mg NH_4Cl /liter of medium

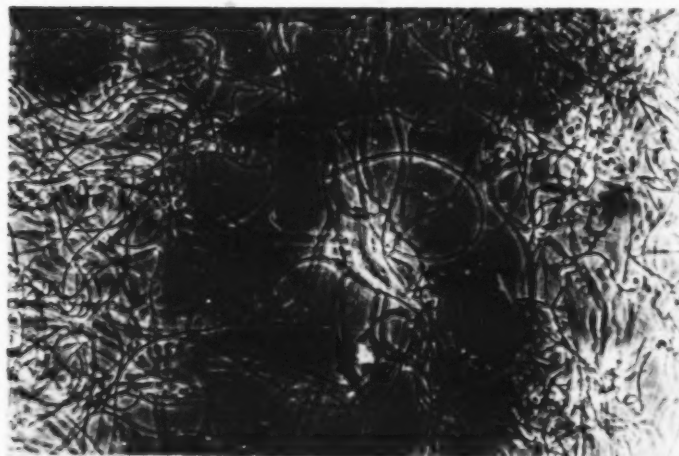


Fig. 7.

Effect of NH_4Cl Concentration and Organic Nitrogen Compounds on the Nature of the Slime Developing in the Continuous Flow Apparatus.

5 mg NH_4Cl /liter of medium



Fig. 8.

Effect of NH_4Cl Concentration and Organic Nitrogen Compounds on the Nature of the Slime Developing in the Continuous Flow Apparatus.

10 mg NH_4Cl /liter and 40 mg Casitone/liter

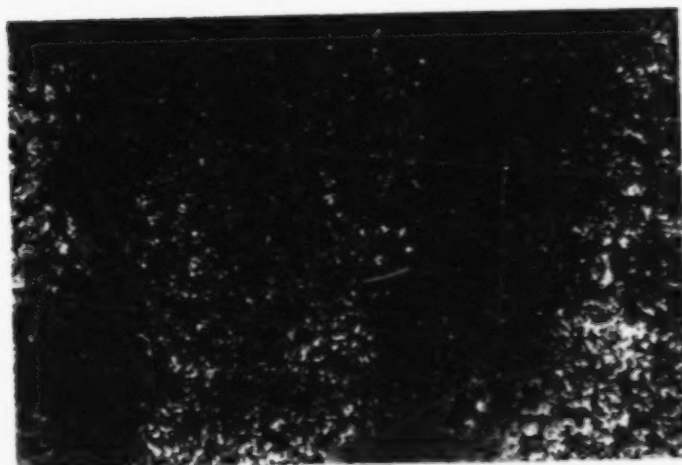


Fig. 9.

Effect of NH_4Cl Concentration and Organic Nitrogen Compounds on the Nature of the Slime Developing in the Continuous Flow Apparatus.

10 mg NH_4Cl /liter and 40 mg Sodium Glutamate/liter

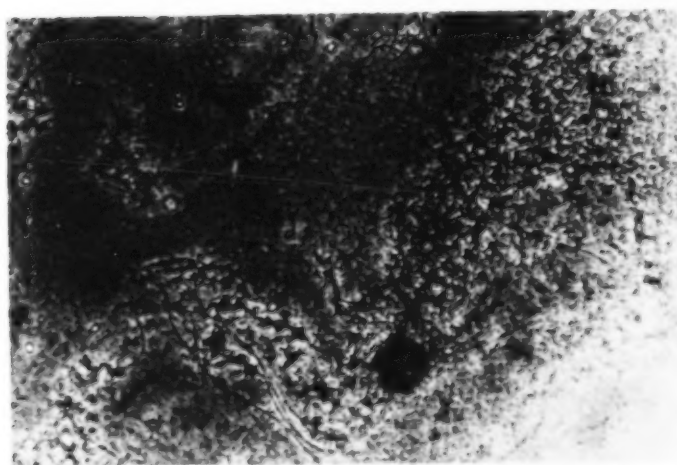


Table 19.

Effect of Glucose Concentration on the Attached Growth of Sphaerotilus natans in the Continuous Flow Apparatus

Glucose Concentration ^a mg/liter	Attached Growth		
	Dry wt. (mg)	Nitrogen	
		(mg)	% (w/w)
10	9.8	1.14	11.6
25	27.8	2.41	8.7
50	38.1	3.11	8.2
100	54.5	4.02	7.4

^a Added to the basal medium containing 10 mg NH_4Cl /liter. The flow rate was 180 ml/hr and 9 liters were passed through each culture vessel.

Table 20.

Effect of Glucose Concentration on the Attached Growth of Mixed Populations in the Continuous Flow Apparatus

Glucose Conc. ^a mg/liter	NH ₄ Cl Conc. ^a mg/liter	Attached Growth		
		Dry wt (mg)	Nitrogen (mg)	% (w/w)
5	1	11.5	1.18	10.3
10	2	14.8	1.56	10.5
10	10	16.8	1.97	11.8
25	5	31.1	3.48	11.2
50	10	55	6.2	11.3
100	20	69.6	7.53	10.9

^a Added to basal medium. The flow rate was 180 mg/hr and 9 liters of medium were fed through each culture vessel.

slimes developed from media containing less glucose appeared to have greater numbers of filamentous Sphaerotilus (Fig. 10 to 12) though, in this case, unlike the nitrogen deficient slimes, the dominance of Sphaerotilus was not overwhelming.

Effect of oxygen concentration: A decrease in the amount of dissolved oxygen from 7.8 mg/liter to 6.9 mg/liter had no influence on the amount of attached growth of Sphaerotilus (Table 21). The amount of growth produced at 1 mg oxygen/liter was greatly reduced. The growth at the reduced oxygen tension comprised of filamentous cells in sheaths that were indistinguishable from cells grown at the higher oxygen tension. The attached growth of mixed populations was reduced when the amount of dissolved oxygen was decreased from 7.8 to 6.9 mg/liter (Table 21). However, the amount of attached growth produced by the mixed population at 1 mg oxygen/liter was greater than that produced by Sphaerotilus alone. Microscopically, the mixed slime developed at 1 mg/liter contained a larger proportion of filamentous Sphaerotilus (Fig. 13) than did the slimes developed at the higher oxygen concentrations (Fig. 14). The slimes developed at 1 mg oxygen/liter did not contain either protozoa or fungi. On the other hand, the slimes growing at the higher oxygen concentrations contained bacteria, protozoa and a fungus. This is the only experiment in the series using synthetic media in which a fungus was selected though, it should be mentioned, the proportion of fungi was not as overwhelming as is indicated in the illustration or was encountered in the experiment using sewage described below. The reason for the selection of the fungus is not clear; the pH of the effluent did not differ from that in other experiments. On repeating this experiment no fungus was encountered though the results with respect to the selection of Sphaerotilus were identical.

Effect of Flow Rate: Pure cultures of Sphaerotilus produced a greater amount of attached growth from 9 liters of medium at lower flow rates (Table 22). However, the rate of growth was slower at the lower flow rates as indicated by the fact that if the attached growth was harvested at about the same time, i.e., less medium had passed through the culture vessels at the lower flow rates, the amount of attached growth was less at the lower flow rate (Table 23). It would therefore appear that after an initial rapid start

Fig. 10.

Effect of Glucose Concentration on the Type of
Slime Developing in the Continuous Flow Apparatus

100 mg glucose/liter of medium



Fig. 11.

Effect of Glucose Concentration on the Type of
Slime Developing in the Continuous Flow
Apparatus

25 mg Glucose/liter of Medium

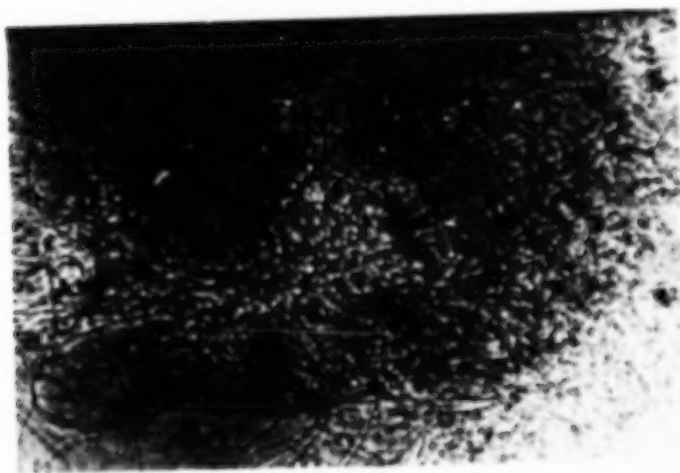


Fig. 12.

Effect of Glucose Concentration on the Type of
Slime Developing in the Continuous Flow Apparatus

5 mg Glucose/liter of medium

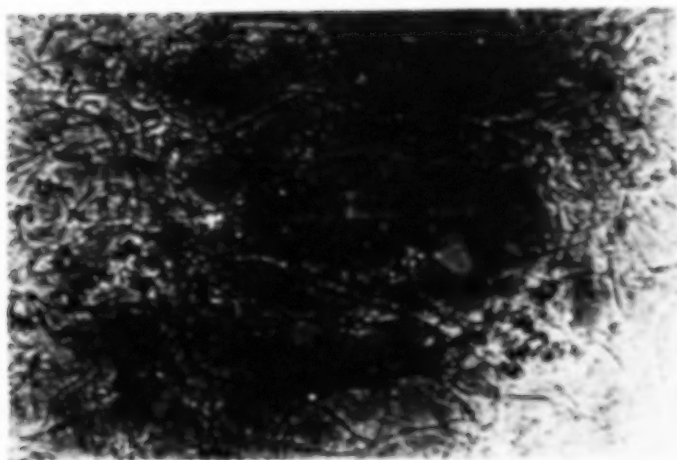


Table 21.

Effect of Oxygen Concentration on the Attached Growth of Sphaerotilus and Mixed Populations

Gas passed through medium ^a	Dissolved oxygen mg/liter	Attached Growth			
		<u>Sphaerotilus</u>		<u>Mixed Populations</u>	
		Dry wt.	Nitrogen	Dry wt.	Nitrogen
		mg	mg	mg	mg
None	6.9	80	5.5	36	4.1
Air	7.8	83	5.5	60	6.2
Nitrogen	1	11	0.7	25	2.7

^a Basal medium containing 10 mg NH_4Cl /liter and 100 mg glucose/liter. The flow rate was 180 ml/hr and each culture vessel received 9 liters of medium.

Fig. 13.

Effect of Dissolved Oxygen Concentration on the
Nature of the Slime Developing in the Continuous
Flow Apparatus.

1 mg Dissolved Oxygen/liter



Fig. 14.

Effect of Dissolved Oxygen Concentration on the
Nature of the Slime Developing in the Continuous
Flow Apparatus.

7.8 mg dissolved oxygen/liter

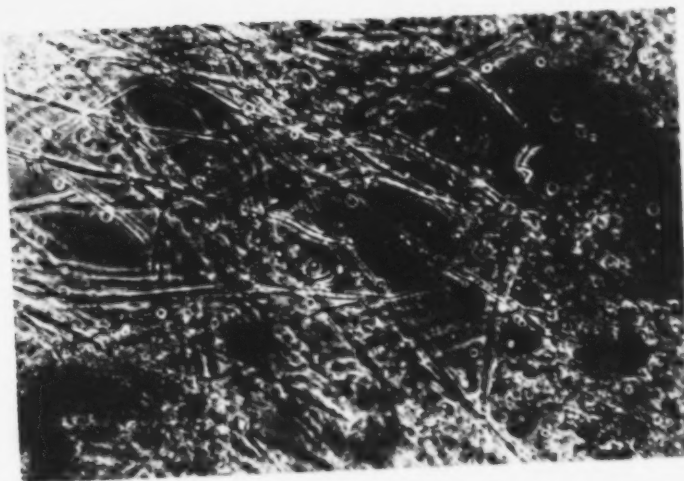


Table 22.

Effect of Flow Rate on the Attached Growth of
Sphaerotilus natans and Mixed Populations in the
 Continuous Flow Apparatus

Flow rate of medium ^a	Attached Growth			
	<u>Sphaerotilus</u>		<u>Mixed Population</u>	
	Dry wt.	Nitrogen	Dry wt.	Nitrogen
ml/hr	mg	mg	mg	mg
250	60.4	4.46	41.6	4.33
180	71.5	4.14	61.7	6.18
90	138.9	10.34	42.6	4.82

^a The medium was composed of the basal medium containing 10 mg NH_4Cl /liter and 100 mg glucose/liter. Nine liters of medium were passed through each culture vessel.

Table 23.

Effect of Flow Rate on the Amount of Attached Growth of Sphaerotilus natans produced in the Continuous Flow Apparatus Fed Varying Amounts of Medium

Flow rate of medium ^a ml/hr	Amount of medium fed through each culture vessel liters	Attached Growth	
		Dry wt. mg	Nitrogen mg
180	9	38.1	3.11
145	7	36.4	3.12
90	4.5	12.5	1.2

^a The medium was made up of the basal medium containing 10 mg NH₄Cl/liter and 50 mg glucose/liter.

the growth rate decreases. We would not expect a steady state to be achieved, however, because of the nature of the growth. We have no experimental data in support or against this suggestion.

In contrast to the above observations on pure cultures the flow rate did not have as great an influence on the attached growth of a mixed population (Table 22). Slightly more growth occurred at a flow rate of 180 ml/hr than at either 90 ml/hr or 250 ml/hr. The lower flow rate, as was expected, allowed a greater quantity of unattached growth; the amount of unattached growth was not estimated. Qualitatively, the slimes produced at the higher flow rates (180 ml/hr and 250 ml/hr) had Sphaerotilus filaments, the higher flow resulting in a larger number of filamentous, Sphaerotilus (Fig. 15). The slime developed at a flow rate of 90 ml/hr was free of Sphaerotilus filaments (Fig. 16).

Experiments with sewage: These experiments were done during the earlier phases of this study and though no quantitative data were recorded they are presented herein as they demonstrate the usefulness of the continuous flow apparatus in the study of microbial ecology. The medium used in this study was settled domestic sewage diluted with water (1/20; 5 day BOD approx. 20 mg/liter) and fortified with glucose (20 mg/liter). The inoculum was activated sludge (10 ml) that contained a fair amount of Sphaerotilus filaments.

The slime developing in the above medium (pH 7.4) had large numbers of Sphaerotilus filaments, protozoa, and zooglycal bacteria (Fig. 17 and 18). When the pH was adjusted to 5.6, Sphaerotilus filaments were no longer evident (Fig. 19). When the pH was decreased to 4.5 the attached slime was dominated by a fungus (Fig. 20), tentatively identified as a Trichosporon species by Wm. B. Cooke. It might be mentioned that attempts to study the growth of this organism in pure culture were not successful inasmuch as the unequal sloughing of the attached growth resulted in poor replicability.

Fig. 15.

Effect of Flow Rate on the Nature of Slime
Developing in the Continuous Flow Apparatus.

250 ml/hr.

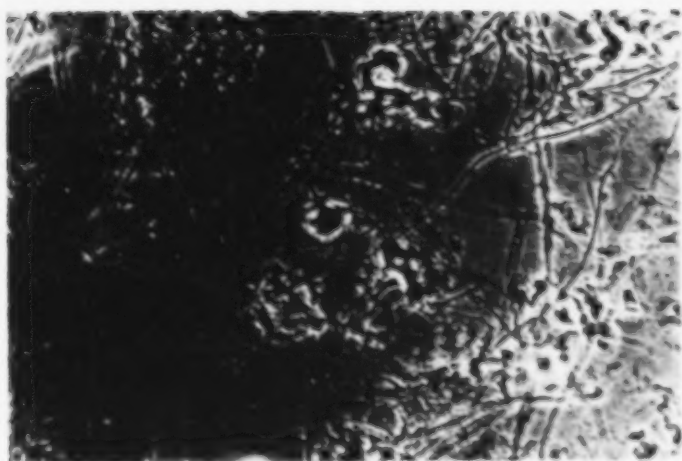


Fig. 16.

Effect of Flow Rate on the Nature of Slime
Developing in the Continuous Flow Apparatus.

90 ml/hr.

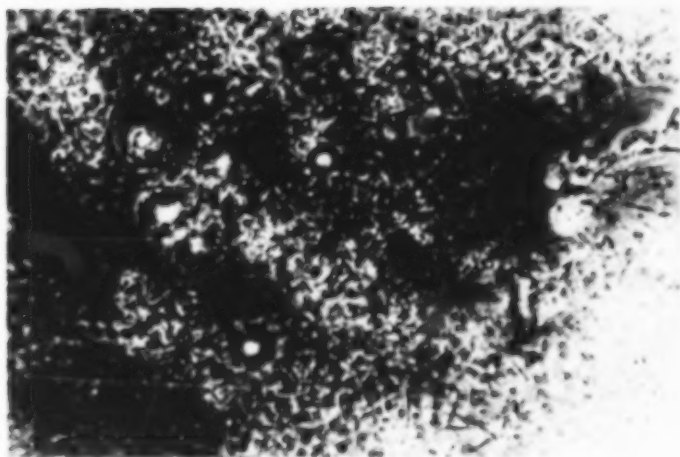


Fig. 17.

Nature of the Attached Growth Developing in the Continuous Flow Apparatus Fed a Sewage Fortified with Glucose and Adjusted to Different pH Values.

pH 7.4

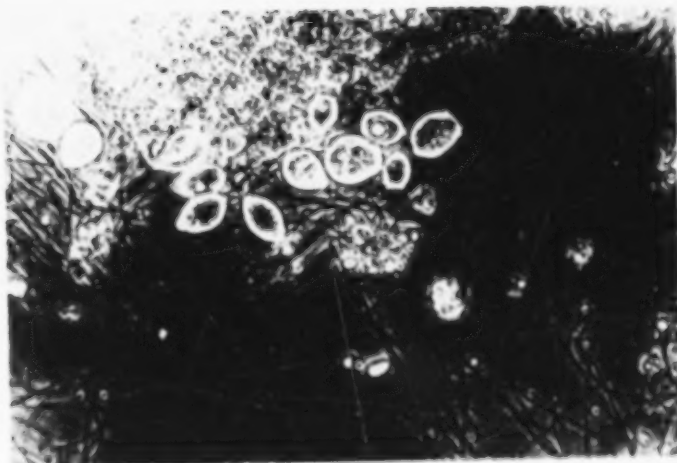


Fig. 18.

Nature of the Attached Growth Developing in the
Continuous Flow Apparatus Fed a Sewage Fortified
with Glucose and Adjusted to Different pH Values.

pH 7.4

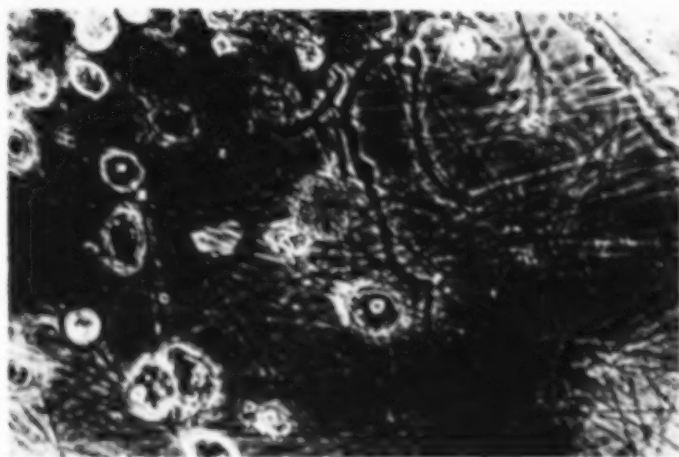


Fig. 19.

Nature of the Attached Growth Developing in the Continuous Flow Apparatus Fed a Sewage Fortified with Glucose and Adjusted to Different pH Values.

pH 5.6

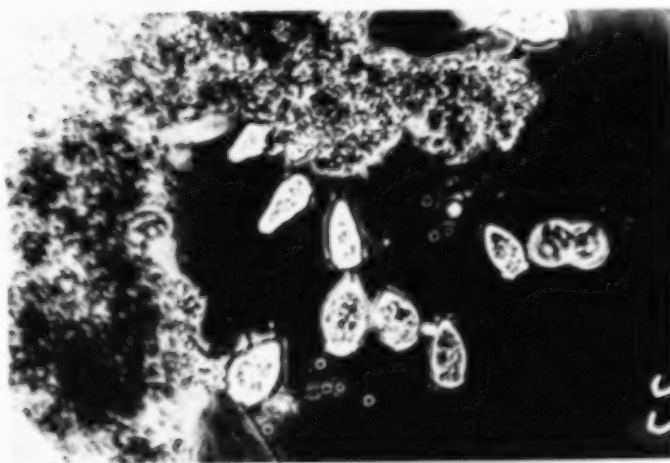


Fig. 20.

Nature of the Attached Growth Developing in the Continuous Flow Apparatus Fed a Sewage Fortified with Glucose and Adjusted to Different pH Values.

pH 4.5



Discussion

Many of the observations recorded for mixed population slimes were expected in the light of earlier reports (12,20,21,23,34,38) on bulking activated sludges. The only unexpected feature was that increased levels of dextrose did not result in the selection of Sphaerotilus. In fact, the opposite seemed to be the case though, in this case, the results were not entirely clear. It is a well documented fact (e.g. 41) that the addition of carbohydrates to normal activated sludge results in Sphaerotilus bulking. Our results indicate that this is probably due to an increased carbon to nitrogen ratio rather than the presence of carbohydrates per se. This has also been indicated by the work of Ingols and Heukelekian (23,24) and that of Hattingh (20). However, it should be noted that Sphaerotilus infestations have also been described under conditions of nitrogen abundance, e.g., streams receiving ammonia-base spent sulphite liquor (3).

Besides the nitrogen deficiency reported herein, Sphaerotilus has also been reported to thrive when the milieu is deficient in phosphorus (20). It is likely that the predilection of Sphaerotilus to dominate under these adverse conditions is a manifestation of the same physiological attribute. What this attribute is is not clear. However, the ability of this organism to accumulate massive amounts of poly- β -hydroxybutyric acid (31,32,36) might have some relevance to the case in point. A much larger amount (dry weight) of growth would be produced at the expense of smaller quantities of either nitrogen or phosphorus. It should be noted though, that several other bacterial species accumulate large amounts of the polyester (8).

The observation that oxygen deficiency selects for Sphaerotilus is probably due to its ability to grow at reduced oxygen tension. The observation that small differences in dissolved oxygen concentrations have no effect on pure Sphaerotilus slimes while greatly affecting the amount of attached growth of a mixed population points in this direction. The results also reveal that very low oxygen concentrations are not conducive to the growth of pure cultures of Sphaerotilus. The observation that very very low oxygen concentrations nonetheless select for Sphaerotilus from a mixed population is possibly an indication that the very low oxygen concentrations are even less favored by the other bacteria that grow attached. The results recorded do not permit one to

decide the minimal oxygen concentration that will allow maximal growth of Sphaerotilus. It might be mentioned that Mulder (32) has reported that Sphaerotilus is not affected by changes in the rate of aeration (shake versus stationary batch cultures) as are certain Arthrobacter species isolated from waste waters.

The experiments with flow rate showed that results obtained with pure Sphaerotilus cultures do not necessarily represent the behavior of Sphaerotilus growing in association with other organisms. Changes in flow rate have a much greater influence on the amount of attached growth of pure Sphaerotilus slimes than they do with mixed population slimes. The reason for this is that at low flow rates and with mixed populations, a large number of organisms grow in an unattached state and are able to compete for food with the attached slime. At higher flow rates the unattached organisms are washed out of the system. However, if the flow rate is increased beyond a certain limit the detention time of nutrients is too small to allow efficient utilization and the amount of attached growth produced is less. At these low detention periods the ability of Sphaerotilus to grow as tassels puts it in a more advantageous position and Sphaerotilus dominates.

The above results on the effect of flow rate on attached growth are at variance with those recorded by Amberg and Cormack (3). This difference is easily explained by the very low nutrient concentrations used in Amberg and Cormack's experiments. Under such conditions one would obtain an increase in attached growth with increased flow rates. Amberg and Cormack's results have greater relevance to conditions in streams and rivers.

The results obtained do not lend support to A.D. Adamse's (1966, Ph.D. thesis, Wageningen, The Netherlands) suggestion that organic nitrogen sources favor the growth of Sphaerotilus. Dias and Heukelekian (11) showed that Sphaerotilus natans utilizes inorganic nitrogen compounds as readily as it does organic nitrogen compounds.

It might be noted that we have not been able to secure identical growth yields in the various experiments even though identical cultural conditions were employed. However, preliminary experiments showed that the coefficient of variation among the 12 culture vessels treated identically was about 10% and, therefore, all conclusions drawn were based on results obtained in the same experiment.

The successful utilization of the continuous flow system is probably the most significant observation of this investigation. Undoubtedly, continuous flow systems have been used to study mixed populations, the most notable probably being those described by Wuhrmann (47). However, the open channels used by most investigators are probably more difficult to control and possibly more expensive to construct and operate. Our continuous flow system is fairly simple and could be used in any microbiology laboratory. It is amenable to the study of pure cultures as well as mixed populations. It should be possible to study not only the sessile bacteria but other organisms, such as stalked ciliate protozoa, as well. A disadvantage of our apparatus is that the medium has a detention time in the culture vessels, a departure from conditions obtaining in the stream.

In conclusion, various environmental conditions are able to select for Spaerotilus from a mixed population. The common occurrence of these organisms is probably a result of the many cultural and nutritional attributes that give the organism a competitive advantage over other aquatic organisms.

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Attached growth of Sphaerotilus and mixed populations in a continuous flow apparatus. F. F. Dias, N. C. Dondero and M. S. Finstein.

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